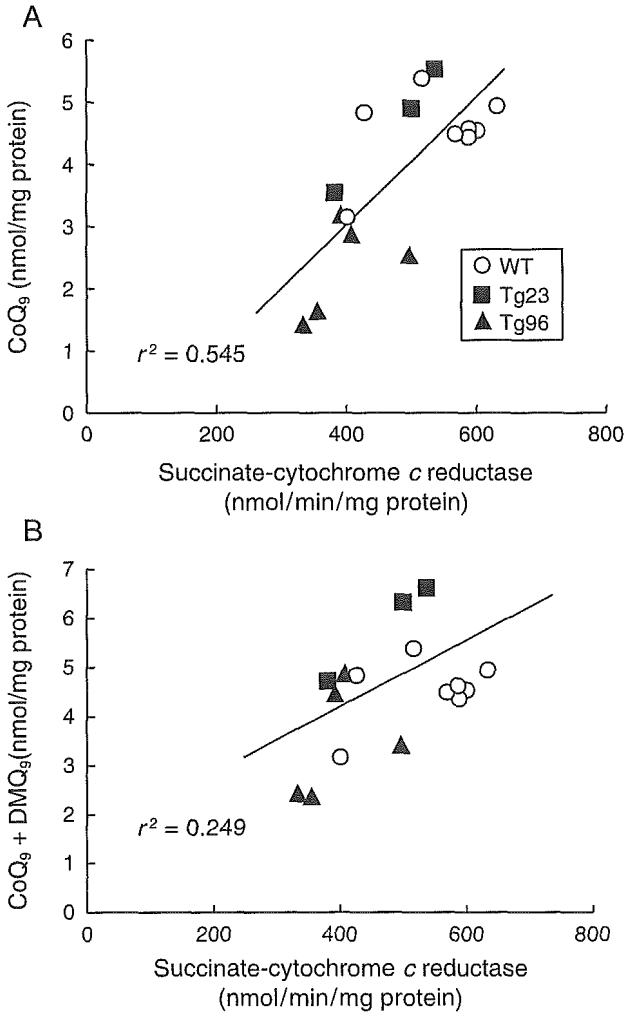

Fig. 5 Correlations between the amounts of quinones and the activities of succinate-cytochrome c redutase in kidney mitochondria in Tg23 and Tg96 mice. (A) A good correlation exists between the activity of succinate-cytochrome c redutase and CoQ_9 content (Pearson's correlation coefficient: $r^2 = 0.545$) (B) However, the amount of $CoQ_9 + DMQ_9$ is weakly correlated with the activity of succinate-cytochrome c redutase ($r^2 = 0.249$).

between succinate-cytochrome c reductase activity and the amount of quinones in kidney mitochondria. Over the physiological range of CoQ_9 content of kidney mitochondria, succinate-cytochrome c reductase activity rises in proportion to CoQ_9 content (Pearson's correlation coefficient; $r^2 = 0.545$, Fig. 5A). However, the amount of $CoQ_9 + DMQ_9$ is weakly correlated with succinate-cytochrome c reductase activity ($r^2 = 0.249$, Fig. 5B). These results indicate that succinate-cytochrome c reductase activity depends on CoQ_9 content in mitochondria of the kidney, whereas DMQ_9 is less likely to contribute to the enzyme activity.

Rescued mice with lower CoQ_9 content generate lower levels of ROS

Because the activities of mitochondrial respiratory enzymes closely correlate with ROS production, we measured the generation of ROS such as $O_2^{\bullet-}$ and H_2O_2 as a deleterious factor of lifespan using the chemiluminescent probes MPEC and DCFH-DA. MPEC and DCFH-DA are primarily sensitive toward $O_2^{\bullet-}$ and H_2O_2, respectively. Kidney mitochondria from Tg96 mice generated less $O_2^{\bullet-}$ (88% of that of wild-type mice) and H_2O_2 (59% of that of wild-type mice) as indicated by the white bars ($P < 0.05$) in Fig. 6(A,B). ROS generation increased in mitochondria when the complex II substrate succinate was added, as previously reported (Liu *et al.*, 2002). When succinate was added, $O_2^{\bullet-}$ (82%) and H_2O_2 (55%) generation from the kidneys of Tg96 was lower than that of wild-type mice (Fig. 6A,B, black bars, $P < 0.05$). These results indicate that mitochondria with a depressed CoQ_9 content generate less ROS.

Discussion

In this study, we successfully rescued COQ7-deficient mice from embryonic lethality by transgenic expression of COQ7 under control of the prion promoter (Fig. 2C). We established two independent rescued lines, Tg23 and Tg96. We detected a comparable level of CoQ_9 in the kidney of Tg23 with that of wild-type mice, whereas Tg96 mice showed less CoQ_9 compared with wild-type mice. In addition, we also detected the definitive DMQ peak, which was identical to that detected in COQ7-deficient mice and *clk-1* in *C. elegans*.

There is an unexplained variance between the expression levels of the COQ7 polypeptide (Fig. 2C) and the level of CoQ_9 (Tables 1 and 2), or the activity of succinate-cytochrome c reductase (Fig. 4) detected in the kidneys from transgenic lines Tg23 and Tg96. That is, Tg96 with the highest gene expression of *clk-1* appears to have the lowest levels of CoQ_9 and the lowest levels of succinate-cytochrome c reductase activity. One possible explanation is that the enhanced expression of COQ7 polypeptide in the kidney of Tg96 may only come from specific renal tubular cells that would not contribute to the overall enzyme activity of succinate-cytochrome c reductase in the kidney. Another possibility is that the protein expression of COQ7 does not necessarily correlate with the enzyme activity in the case of exogenously expressed COQ7 in transgenic mice.

CoQ is an electron transporter between complex I and complex III, or between complex II and complex III. To understand the influence of depressed CoQ on mitochondrial functions, we measured the activities of respiratory chain enzymes in Tg23 and Tg96 mice. We observed the depressed enzyme activities of CoQ-responsive respiratory chain in the isolated mitochondria from the kidney of Tg96 (Fig. 4A), indicating that succinate-cytochrome c reductase activity significantly correlates with CoQ_9 contained in the mitochondria (Fig. 5). Human cases with CoQ_{10} deficiency have been reported, and these patients usually develop symptoms of mitochondiral encephalomyopathy from early childhood (Ogasahara *et al.*, 1989; Boitier *et al.*, 1998; Sobreira *et al.*, 1997; Di Giovanni *et al.*, 2001). They showed depressed levels of CoQ_{10} in muscle, ranging from 4% to 39% of healthy controls, as well as depressed NADH– and succinate-cytochrome c reductase activities. Interestingly,

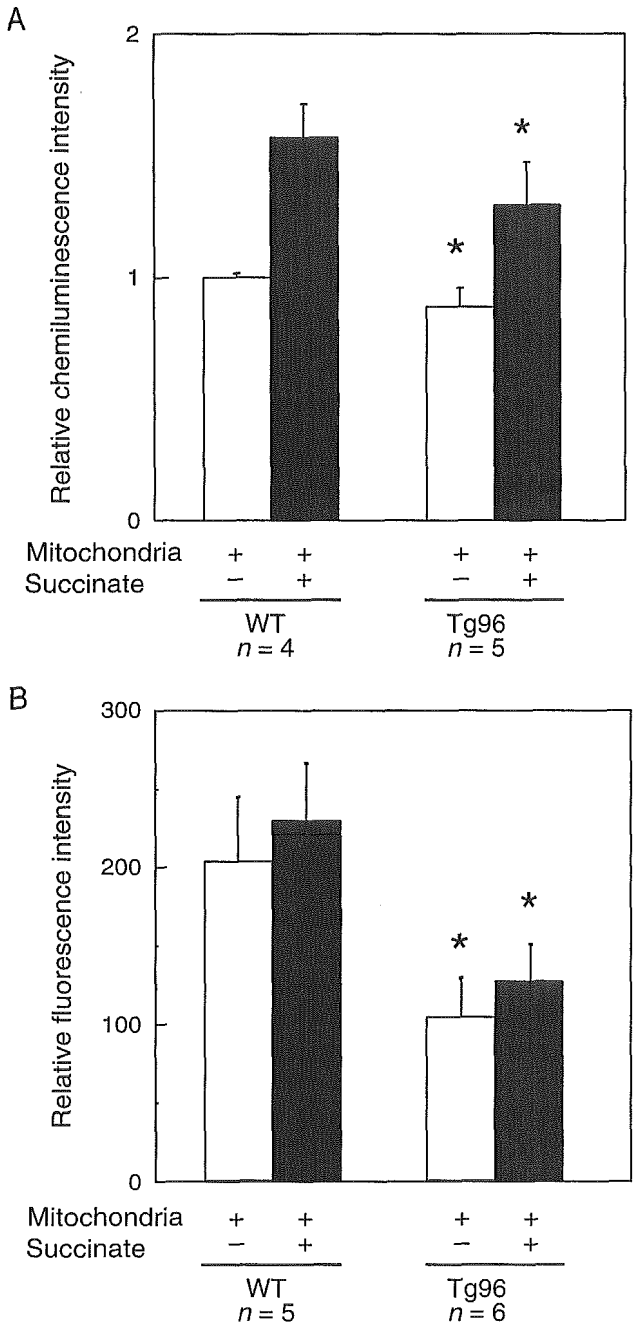

Fig. 6 Reduced generation of reactive oxygen species (ROS) in kidney mitochondria of rescued COQ7-deficient mice. (A) Mitochondrial $O_2^{\bullet-}$ generation in the kidneys of Tg96 mice. Using the chemiluminescent probe MPEC, $O_2^{\bullet-}$ generation from wild-type and Tg96 mice was measured in the presence or absence of 1.5 mM succinate. Error bars represent the standard deviation. On each experimental day, each value was calculated on the basis of the average of the chemiluminescence intensity of wild-type mice in the presence of 100 μg of mitochondria only (white bar). With mitochondria only (white bars, $*P < 0.05$) or with succinate as a substrate (black bars, $*P < 0.05$), kidney mitochondria from Tg96 showed lower generation than that of wild-type mice. (B) Mitochondrial H_2O_2 generation was also measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as a fluorometric probe. The emitted fluorescence from this probe is directly proportional to the concentration of H_2O_2 (Wang & Joseph, 1999). The relative fluorescence intensity from wild-type and Tg96 mice was measured in the presence or absence of 2 mM succinate. Error bars represent the standard deviation. For all experimental conditions, kidney mitochondria from Tg96 showed lower H_2O_2 generation than that of wild-type mice ($*P < 0.05$).

succinate-cytochrome *c* reductase activity was also slightly depressed in *clk-1* mutants (Felkai *et al.*, 1999; Miyadera *et al.*, 2001), whereas it was severely depressed in embryonic stem (ES) cells derived from embryos of COQ7-deficient mice (Levavasseur *et al.*, 2001). The *clk-1* mutant worms absorb CoQ_8 from dietary *E. coli* (Jonassen *et al.*, 2001) while endogenously generating DMQ_9 in its mitochondria (Miyadera *et al.*, 2001). COQ7-deficient ES cells, however, only represent DMQ_9 in their mitochondria (Levavasseur *et al.*, 2001). Taken together, our results suggest that DMQ_9 has only a minor physiological role as an electron transporter in mitochondria. In support of this idea, a DMQ derivative shows a depressed potential for transporting electrons compared with the CoQ derivative *in vitro* (Gu *et al.*, 1990). Alternatively, DMQ_9 may possibly compete with CoQ_9 in the mitochondrial respiratory chain if DMQ_9 and CoQ_9 coexist in the same mitochondria. In this case, DMQ has an inhibitory effect on the electron transfer in oxidative phosphorylation as well as on the generation of ROS.

The domains of NADH- and succinate-cytochrome *c* reductase are suggested as the main sites of $O_2^{\bullet-}$ generation (Chance *et al.*, 1979; Turrens *et al.*, 1985). In addition, the ubiquinone binding site in complex III and the flavin mononucleotide group binding site in complex I have been also reported to be ROS-generating sites (Turrens & Boveris, 1980; Turrens *et al.*, 1985). It is thus noteworthy that the rate of $O_2^{\bullet-}$ generation in cardiac submitochondrial particles is directly related to the relative amount of CoQ_9 (Lass *et al.*, 1997). These reports are therefore consistent with data demonstrating that Tg96 mice showed a depressed rate of $O_2^{\bullet-}$ generation from kidneys (Fig. 6), which concomitantly showed the depressed enzyme activities of CoQ-responsive respiratory chain (Figs 4 and 5). In addition, Tg96 mice generated significantly lower amounts of H_2O_2 than wild-type mice, and they also generated slightly lower amounts of $O_2^{\bullet-}$ (Fig. 6). Because $O_2^{\bullet-}$ generated in the mitochondria is rapidly converted to H_2O_2 by endogenous manganese superoxide dismutase (Mn-SOD), it is difficult to detect *de novo* $O_2^{\bullet-}$ accurately (Forman & Azzi, 1997). It is reported that SOD activity in the *clk-1* mutant is lower than that of the wild-type (Braeckman *et al.*, 2002). We assumed that lower amounts of CoQ_9 in Tg96 mice limit the rate of electron flow and regulate respiratory chain enzyme activities at the CoQ site in the mitochondrial respiratory chain. We consider that retarded electron flow influences the rate of electron leakage and $O_2^{\bullet-}$ generation.

The mechanism by which *clk-1* extends lifespan is still to be defined. Recently, RNA interference (RNAi) on the genes for the respiratory complex (Dillin *et al.*, 2002) as well as the genes involved in CoQ synthesis (Asencio *et al.*, 2003) such as *clk-1* extend lifespan in *C. elegans*. These data indicate that the extension of animal lifespan might be attributed to the depressed activities of mitochondrial enzymes (Dillin *et al.*, 2002), or alternatively to the suppressed generation of superoxide caused by depressed CoQ (Asencio *et al.*, 2003). Contrary to the latter hypothesis, Braeckman *et al.* (2002) reported that the generation of ROS in *clk-1* (*e2519*) was slightly elevated when measured using lucigenin luminescence.

In this study, we established a novel mammalian model system to investigate the relationship between *coq7/clk-1* and mitochondrial functions. Our COQ7-deficient model mice with a COQ7 transgene represent both DMQ and CoQ in mitochondria. The lower amounts of CoQ_9 in this model may cause a decrease in the enzyme activities of the mitochondrial respiratory chain as well as the decrease in ROS generation. Thus, an animal model carrying variable amounts of quinones in mitochondria would be useful for the analysis on the mechanism of lifespan that is controlled by mitochondrial functions.

Experimental procedures

Generation of COQ7 transgenic (Tg) mice

A COQ7/CLK-1 Tg construct was assembled by inserting mouse *coq7* cDNA into the *Xho*I site of the transgenic vector MoPrP.Xho (Borchelt *et al.*, 1996). After linearization with *Not*I, the Tg construct was isolated by gel electrophoresis and purified using a Gene Clean II Kit (Bio 101, Vista, CA, USA). Purified Tg construct was then microinjected into the pronuclei of BDF1 mice (Nihon SLC, Shizuoka, Japan). Tail DNA from potential founder mice was screened by PCR amplification with a sense primer (5′-GTGGGATCAAGAGAAGAACC-3′) and antisense primer (5′-AGAAGCAAGAATGAGAACCACCTC-3′). Four lines of germline mice transmitted Tg, Tg23, Tg82, Tg95 and Tg96, and were selected for further analysis. To generate COQ7 Tg mice with a *coq7*–/– background, we crossed *coq7*+/– mice with COQ7 Tg mice. In all four lines, the transgene was successfully transmitted to the germline of F1 mice. Subsequently, *coq7*+/– mice expressing the COQ7 transgene were crossed with *coq7*+/– mice to generate COQ7 Tg mice with a *coq7*–/– background. Finally, two lines of COQ7 Tg mice (Tg23 and Tg96) successfully rescued the embryonic lethality of COQ7-deficient mice.

Preparation of anti-mouse *coq7/clk-1* antibody

A 537-bp fragment containing mouse *coq7/clk-1* cDNA fragment (39–217 amino acid residues) starting from the second ATG was PCR-amplified with primers (*Not*I-anchored primer: 5′-ATAAGAATGCGGCCGCACCTTAGACAATATTAACCGGG-3′ and *Bam*HI-anchored primer: 5′-CGGGATCCAAACCTTTCTGA-TAAATA-3′) from the plasmid pcDNA3.1 (Invitrogen, CA, USA) containing full-length mouse *coq7/clk-1* cDNA (GeneBank Accession number AF098949) (Takahashi *et al.*, 2001). A *Not*I/*Bam*HI-restricted PCR product was inserted into a *Not*I/*Bam*HI-restricted pIVEX2.4a vector (Roche, Mannheim, Germany) for *in vitro* translation. Recombinant mouse *coq7/clk-1* (39–217) proteins were synthesized at 30 °C for 24 h at 120 r.p.m. stirring using a Rapid Translation System RTS500 *E. coli* HY kit (Roche). The N-terminal His-tagged proteins were precipitated by incubation and collected by centrifugation. The pellet was washed with phosphate-buffered saline (PBS) and dissolved into urea lysis buffer (8 M urea, 20 mM sodium phosphate (pH 7.8), and 500 mM NaCl). The urea-soluble fraction was purified by an Ni^{2+}-charged HiTrap chelating HP column (Amersham Pharmacia Biotech, Uppsala, Sweden). After dialysis against PBS, the purified protein was injected three times into a New Zealand white rabbit with Freund's complete or incomplete adjuvant. A week after the last injection, antiserum was collected and the IgG fraction was purified using a HiTrap protein G HP column (Amersham Pharmacia Biotech).

Western blotting of COQ7

Mouse tissues were homogenized in 10 mM sodium phosphate (pH 7.2), 1 mM EDTA, 0.2 mM pheneylmethylsulphonyl fluoride and 0.5 μg mL^{-1} leupeptin containing 1% Triton X-100. The homogenates were centrifuged at 20 000 ***g*** for 10 min at 4 °C and the supernatants were subjected to Western blotting. Procedures of Western blotting were as previously described (Takahashi *et al.*, 2001) except for the use of anti-mouse *coq7/clk-1* antibody.

Analysis of coenzyme Qs (quinones)

Quinones were extracted from tissue homogenates by ethanol/*n*-hexane (2 : 5, v/v) as previously described (Nakai *et al.*, 2001) and subjected to reverse-phase high-performance liquid chromatography (HPLC) (Devolosil C-30-UG-5 3.0 × 150 mm, Nomura Chemical, Aichi, Japan). Quinones were eluted by ethanol/methanol (1 : 1, v/v) at 0.43 mL min^{-1}. The amount of CoQ_9 or CoQ_{10} was determined by comparing them with CoQ_9 and CoQ_{10} standards (Sigma, St Louis, MO, USA), respectively. The amounts of DMQ_9 were also calculated by comparing them with CoQ_9 standards.

Mitochondrial isolation

Mitochondria were isolated from the supernatants by differential centrifugation (Lash & Sall, 1993). Mouse kidney or liver was homogenized in 10 volumes (w/v) of Buffer-1 (0.25 M sucrose, 0.2 mM EDTA buffer) using a Teflon pestle with a 0.3-mm clearance by nine up and down strokes at 1200 r.p.m. on a Digital homogenizer HOM (Asone, Osaka, Japan). All operations were performed at 4 °C. The homogenates were centrifuged at 100 ***g*** for 5 min. After a 0.5 volume of Buffer-2 (0.35 M sucrose, 0.2 mM EDTA) was added to each supernatant, the solutions were centrifuged at 800 ***g*** for 15 min. The supernatants were then centrifuged at 9000 ***g*** for 10 min and the pellets were resuspended in the same volume of Buffer-1. The suspensions were washed at 9000 ***g*** for 7 min and the mitochondrial fraction was resusupended at a final concentration of 10–15 mg mitochondrial protein/mL in Buffer-1. Protein concentration was determined using a DC protein Assay kit (Bio-Rad, CA, USA) according to the manufacturer's instructions.

Analysis of respiratory chain enzyme activities

Mitochondrial fractions of kidney and liver were stored at −30 °C for up to 1 month. Succinate-cytochrome c reductase activity (complex II + III) was measured as follows; the reduction of cytochrome c by complex III coupled to succinate oxidation through complex II was followed at 550–540 nm (extinction coefficient of 19.0 mM cm^{-1}) using a double-beam spectrophotometer, DU7500 (Beckman, CA, USA). In a 1-mL cuvette, a reaction mixture consisting of 40 mM potassium phosphate (pH 7.4), 20 mM succinate, 0.5 mM EDTA, 2 mM KCN and 100 μg kidney or liver mitochondrial protein was incubated at 30 °C for 20 min. The reaction was initiated by adding 30 μM cytochrome c and the reduction of cytochrome c was monitored as a linear absorbance increase for 3 min (Trounce *et al.*, 1996).

Determination of the generation of ROS in mitochondria

Superoxide anion generation was measured using the chemiluminescent probe MPEC (2-methyl-6-p-methoxyphenylethynylimidazopyrazinone) (ATTO, Tokyo, Japan). MPEC is a new imidazopyrazinone derivative that is sensitive and useful for measuring superoxide (Shimomura *et al.*, 1998). Mitochondrial proteins (100 μg) were added to 1 mL of assay buffer (50 mM HEPES (pH 7.4), 2 mM EDTA) containing 0.05 mM MPEC. The reaction mixture was placed in a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany) and the chemiluminescence intensity of MPEC was measured for 1 min at room temperature. To measure superoxide anion generation from mitochondria with a respiratory substrate, 1.5 mM succinate as a substrate of respiratory complex II was added to the solution. The absolute chemiluminescence intensity used to quantify superoxide anion generation was calculated by subtracting the background intensity (in the absence of mitochondria). The experiments were repeated three times. Data analysis was performed as follows: the relative chemiluminescence intensity was calculated for each experimental day using the formula [chemiluminescence intensity for each condition/the average chemiluminescence intensity of the wild-type in the presence of 100 μg of mitochondria without substrate].

ROS including H_2O_2 were also measured by 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes, OR, USA), which was used as a fluorometric probe. When DCFH-DA crosses the mitochondrial membrane, it is cleaved to non-fluorescent DCFH by an esterase (Bejma & Ji, 1999). Furthermore, DCFH is oxidized to fluorescent dichlorofluorescin (DCF) by intramitochondrial ROS. The fluorescence emitted is directly proportional to the concentration of H_2O_2 (Wang & Joseph, 1999). In our experiment, mitochondrial proteins (50 μg) were added to 1 mL of assay buffer (130 mM KCl, 5 mM $MgCl_2$, 20 mM Na_2HPO_4, 20 mM Tris-HCl, 30 mM glucose (pH 7.4)) containing 5 μM DCFH-DA with or without 2 mM succinate. The reaction mixture was incubated at 37 °C for 15 min, and then centrifuged at 12 000 **g** for 8 min to discard supernatant containing DCFH-DA that did not cross the mitochondrial membrane. The mitochondrial pellets were resuspended in the assay buffer. DCF formation was quantified at an excitation wavelength of 488 nm and emission wavelength of 525 nm using a Spectra Max Gemini XS fluorescent microplate reader (Molecular Devices, CA, USA) at 37 °C. The experiments were repeated three times.

Statistical analysis

Data on CoQ content, mitochondrial respiratory chain enzyme activity and relative chemiluminescence intensity of MPEC were separately analysed using Student's *t*-test.

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Development of a geriatric autopsy database and Internet-based database of Japanese single nucleotide polymorphisms for geriatric research (JG-SNP)

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The GEAD and JG-SNP have been created by joint collaboration between the Tokyo Metropolitan Geriatric Medical Center and the Japan Science and Technology Agency (JST)

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Abstract

To facilitate geriatric research on the roles of genetic polymorphisms of candidate genes, two databases were developed based on data obtained from autopsy examinations of elderly subjects: the geriatric autopsy database (GEAD) and the Japanese single nucleotide polymorphisms (SNP) database for geriatric research (JG-SNP) which is accessible on the Internet (http://www.tmgh.metro.tokyo.jp/jg-snp/english/E_top.html). The data for the GEAD were derived from 1074 consecutive autopsy cases (565 male and 509 female cases) with an average age of 80 years. The GEAD was installed on a stand-alone Windows 2000 server using Oracle 8i as the database application. The GEAD contains clinical diagnoses of 26 geriatric diseases, histories of smoking and alcohol consumption, pathological findings (720 items), severity of atherosclerosis, genetic polymorphism data, etc. On the JG-SNP website, case distribution corresponding to a specified SNP or disease can be searched or downloaded. Although there are several Internet-based SNP databases such as dbSNP, no databases are available at present on the web that contain both SNP data and phenotypic data. As autopsy studies can provide large amounts of accurate medical information, including the presence of undiagnosed diseases such as latent cancers, the GEAD is a unique and excellent database for research on genetic polymorphisms.

Keywords: Geriatrics; Pathology; Autopsy; Genetic polymorphism; Single nucleotide polymorphism; Database

Abbreviations: ACE, angiotensin-converting enzyme; DHPLC, degenerative high performance liquid chromatography; GEAD, geriatric autopsy database; JG-SNP, Japanese SNP database for geriatric research; PCR-RFLP, polymerase chain reaction restriction fragment length polymorphism; PON1, paraoxonase 1; SNP, single nucleotide polymorphism.

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1. Introduction

Improvements in health care management have increased the population of the aged in Western and some other countries. Geriatric medicine is thus becoming an important medical field. Most geriatric diseases are multifactorial diseases and are considered to be caused by interactions between hereditary factors such as disease susceptibility of the individuals and external factors such as smoking. The susceptibility and resistance to a particular disease show a wide range of individual differences that are believed to stem from subtle differences of the genome called genetic polymorphism. Genetic polymorphisms, in turn, can be used to identify the genes responsible for diseases (Schneider et al., 2003).

Tokyo Metropolitan Geriatric Medical Center is one of the institutions in Japan where autopsy examination is conducted very frequently. Over the last three decades, the center has performed more than 7000 autopsies, and has registered all the clinical and pathological information collected thus in a pathology database named "ANATOMY" (Ohtsubo et al., 1992). Based on this database, the authors have been studying the relationships between geriatric diseases and genetic polymorphisms of candidate genes. We have already reported associations between an insertion/deletion polymorphism of the angiotensin-converting enzyme (ACE) gene and cardiomegaly, a single nucleotide polymorphism (SNP; the most frequent type of genetic polymorphism) of the paraoxonase 1 (PON1) gene and Parkinson's disease, and a SNP of the estrogen receptor-alpha gene and Alzheimer's disease (Nakahara et al., 2000; Kazama et al., 2002; Kazama et al., 2004).

The objective of the present study was to establish an autopsy database specifically designed to promote further genetic researches on the relationships between geriatric diseases and genetic polymorphisms. The geriatric autopsy database (GEAD) established by the authors contains clinicopathological information and SNP data derived from autopsy examination of elderly subjects. Based on the GEAD, we have developed another database named "Japanese SNP database for geriatric research (JG-SNP)," which is accessible to the public on the Internet. Researchers interested in genetic polymorphisms can access our database on the web to obtain the allele frequencies of SNPs of candidate genes and associations between SNPs and geriatric diseases.

2. Materials and methods

2.1. Materials

The subjects were 1074 consecutive autopsy cases performed at the Tokyo Metropolitan Geriatric Medical Center during the last 5.5 years. They consisted of 565 males and 509 females, and the average age (±S.D.) of the subjects was 79.2 (±8.1) years for males and 81.8 (±9.5) years for females. The subject population also included 167 nanogerians and 7 centenarians. The average autopsy rate at this center during this period was 40%.

2.2. Flow and control of clinicopathological information

At autopsy, all internal organs were extirpated, examined, and fixed in 10% formalin. Two or three weeks after autopsy, macroscopic re-examinations of the extirpated organs were performed at weekly gross conferences. With the results of microscopic examinations, all cases were presented and discussed in details with clinicians at weekly autopsy conferences. All initial data were taken from the gross and microscopic autopsy reports and entered into the free-text type autopsy database, ANATOMY (Ohtsubo et al., 1992). The data on ANATOMY were then translated into the GEAD by the pathologists (M.S., T.A. and I.K.). The GEAD has been installed on a stand-alone Windows 2000 Server, and Oracle 8i, R.8.1.6 (Oracle Corp. Japan, Tokyo) was employed as the database application. The statistical analysis on the data of the GEAD was performed by one of us (N.T.) using the SAS system for Windows, V8 (SAS Institute Japan Ltd., Tokyo). The JG-SNP was installed on an Internet server run by NTT Hokkaido Telemart Co. Ltd., Sapporo, Japan.

2.3. Preparation and Storage of DNA

At the time of autopsy, small specimens from each of the five following organs, namely, the renal cortex, liver, ventricular myocardium, esophageal mucosa, and cerebral cortex were obtained and stored frozen at −80 °C. Brain specimens were obtained in 84% of the cases. Serum specimens collected within a week prior to death were also available for 75% of the cases. DNA used for genetic analysis was extracted from the renal cortex by the phenol–chloroform method, and stored frozen until use.

2.4. Genotyping assays

Depending on each polymorphic site of the candidate genes, one of the three genotyping methods was applied; the degenerative high performance liquid chromatography (DHPLC), polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), and Taqman method. When none of these methods was applicable, direct sequencing was performed for genotyping. All exons including exon–intron junctions and promoter regions of the candidate genes were amplified by PCR and analyzed by the DHPLC to detect genetic polymorphisms. The DHPLC was performed by the Transgenomic WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NE, USA) according to the manufacture's instructions. When more than one peaks appeared on the chromatography, these amplicons were directly sequenced. If restriction sites were present at

the polymorphic sites, PCR-RFLP was a choice of analysis. The allelic discrimination assay using Taqman fluorogenic probes was performed by ABI Prism 7000 according to the manufacture's instructions. All polymorphic data were validated by the direct sequencing of the PCR amplicons. The direct sequencing was performed by either ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA, USA) or GeneRapid (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The detailed methods of analysis on each SNP will appear on the web pages of SNP data.

2.5. Ethical considerations

Written informed consent was obtained from the bereaved family of each of the patients prior to the autopsy examination. The use of autopsy materials for medical education and research is generally permitted by the Act of Postmortem Examinations of Japan. The database server and software have their own user IDs and passwords to protect the data from unauthorized access. The GEAD also has a provision to provide anonymity, in which a new ID is assigned to the patient ID to conceal the identity in cases of the leakage of data. Approvals for individual genetic researches and the release of the JG-SNP to the public on the Internet were obtained from the Ethical Committee of the Tokyo Metropolitan Geriatric Medical Center.

3. Results

3.1. Two database systems

The autopsy subjects of the GEAD were a specific group from our center, and the number of registered cases was as small as 1074 cases. The GEAD contains highly confidential, personal information, such as disease profile of the subjects. Accordingly, maximum caution to prevent leakage of confidential information is required before releasing the database to the public. Therefore, we established another separate database, namely JG-SNP, for Internet users. The flow-chart of the databases is shown in Fig. 1.

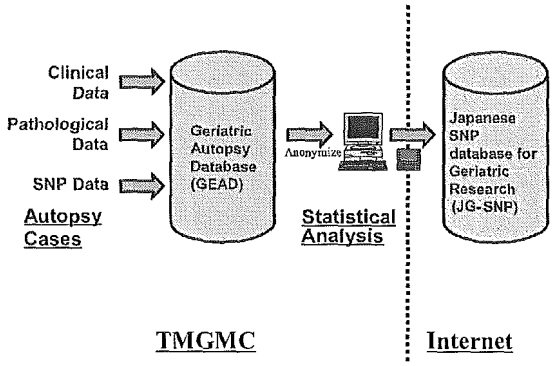

Fig. 1. Flow-chart of information compiled into GEAD and JG-SNP.

3.2. Geriatric autopsy database (GEAD)

The GEAD contains the following items:

1. Patient information: gender, date of birth, date of death, age at the time of death, and postmortem time to autopsy.
2. Clinical information: presence or absence of 26 geriatric diseases, results of clinical dementia rating, and histories of smoking and alcohol consumption. The 26 geriatric diseases included the following diseases/pathological conditions: ischemic heart disease, atrial fibrillation, degenerative valvular diseases, hypertension, aneurysm, arteriosclerosis obliterans, dementia, cerebrovascular disorder, Parkinson's disease, diabetes mellitus, hyperlipidemia, malnutrition, osteoporosis, degenerative osteoarthritis, aspiration, chronic obstructive pulmonary disease, idiopathic interstitial pneumonia, urinary tract infection, prostatic hypertrophy, decubital ulcer, lung cancer, gastric cancer, colon cancer, hematopoietic malignancy, cataract, and glaucoma.
3. Pathological findings: systemic pathological findings consisted of 720 items. The 720 codes used herein were originally created from pathological findings frequently encountered in autopsy examinations of elderly subjects. The data were entered as semi-quantitative values in the database. A code table of pathological findings of the heart is shown as an example in Table 1. Also included were the severity of atherosclerosis and pulmonary emphysema, degree of coronary arterial stenosis, weights of the organs, and volumes of intracavitary fluids.
4. Genetic polymorphism data: the current data included insertion/deletion polymorphism of the ACE gene and SNP of the PON1 gene. A total of 29 SNP data of the 13 candidate genes shown below are scheduled to be open at the next revision of the web pages: ACE, ALPL (alkaline phosphatase, liver/bone/kidney), CYP2C9 (P450, family 2, subfamily C, polypeptide 9), ESR1 (estrogen receptor-alpha), GGCX (gamma-glutamyl carboxylase), IL6 (interleukin 6, interferon, beta 2), KL (klotho), MTHFR (5,10-methylenetetrahydrofolate reductase, NADPH), NOS3 (nitric oxide synthase 3, endothelial cell), PON1, PPARG (peroxisome proliferative activated receptor, gamma), WRN (Werner syndrome), ZNF147 (zinc finger protein 147, estrogen-responsive finger protein). Additional 20 SNPs are scheduled to be open in the near future.

3.3. Japanese SNP database for geriatric research (JG-SNP)

The following items are included in the JG-SNP:

1. Patient information: gender and age group at the time of death.
2. Clinical diagnosis: presence or absence of the above-mentioned 26 geriatric diseases.

Table 1
Code table for pathological findings of the heart

Code number	Pathological diagnosis
1	Miscellaneous
2	Atrial septal defect
3	Ventricular septal defect
4	Bicuspid aortic valve
5	Endocarditis, NOS
6	Nonbacterial thrombotic endocarditis
7	Infectious (bacterial) endocarditis
8	Thrombosis
9	Atrial thrombosis
10	Tricuspid regurgitation (TR)
11	TR with ring-dilatation (relative TR)
12	Pulmonary valvular regurgitation
13	Mitral regurgitation (MR)
14	Mitral stenosis (MS)
15	Mitral stenosis and regurgitation (MSR)
16	Mitral-ring calcification (MRC)
17	MR with ring-dilatation (relative MR)
18	Mitral valve prolapse (hooding)
19	MR with papillary muscle dysfunction
20	MR with rupture of the chordae tendineae
21	Aortic (valvular) regurgitation (AR)
22	Aortic (valvular) stenosis (AS)
23	Calcification of the aortic valve
24	Aortic stenosis and regurgitation (ASR)
25	Degenerative valvular diseases
26	Degenerative aortic valvular diseases
27	Degenerative mitral valvular diseases
28	Rheumatic valvular diseases
29	Status after valvular surgery
30	Myocardial infarction (MI)
31	Acute myocardial infarction (AMI)
32	Old myocardial infarction (OMI)
33	OMI with acute extension
34	MI with thrombosis
35	MI with ventricular aneurysm
36	MI with ventricular/septal rupture
37	Myocardial fibrosis/scars, NOS
38	Myocardial necrosis, NOS
39	Coronary arteriosclerosis
40	Status after coronary angioplasty or surgery
41	Amyloidosis or amyloid deposition
42	Myocarditis, NOS
43	(Brown) atrophy
44	Left ventricular hypertrophy
45	Left ventricular dilatation
46	Right ventricular hypertrophy
47	Right ventricular dilatation
48	Cor pulmonale
49	Atrial dilatation (atriomegaly)
50	Cardiomyopathy
51	Hypertrophic cardiomyopathy
52	Dilated cardiomyopathy
53	Metastatic tumors
54	Status after pacemaker implantation
55	Cardiac tamponade
56	Pericarditis, NOS
57	Purulent pericarditis, NOS
58	Hemorrhagic pericarditis, NOS
59	Fibrous/fibrinous pericarditis, NOS
60	Pericarditis carcinomatosa

3. Pathological findings: presence or absence of 12 pathologically-identified diseases/pathological conditions, including atherosclerosis, gastric ulcer, pneumonia, cholelithiasis, urinary tract infection, and cancers of seven organs (thyroid, lung, stomach, colon, liver, kidney, and prostate).
4. Genetic polymorphism data: genetic polymorphism data of ACE and PON1. Also included in the homepage of the JG-SNP are characteristics of geriatric diseases, outlines of genetic polymorphisms, brief explanations of each geriatric disease, research results on genetic polymorphisms studied by us, ethical considerations, call for joint study, etc.

The major functions of the JG-SNP are outlined as follows:

1. Case distribution of genetic polymorphisms according to the clinical and pathological diagnoses can be searched and downloaded in a comma separated value (CSV) format.
2. Case distribution of clinical and pathological diagnoses corresponding to a specified genetic polymorphism can be searched and retrieved.
3. Gender and age group of the cases can be specified on search.
4. In order to prevent leakage of confidential information during any searches on the web, if there is only one case corresponding to a specific SNP and a specific disease, the JG-SNP is so constructed that the data will not be displayed on the screen. The corresponding data are excluded from the total counts of the table as well.

The followings are the URLs for the home page of the JG-SNP. The "Search by Disease" screen of the JG-SNP home page is shown in Fig. 2. English version: http://www.tmgh.metro.tokyo.jp/jg-snp/english/E_top.html. Japanese

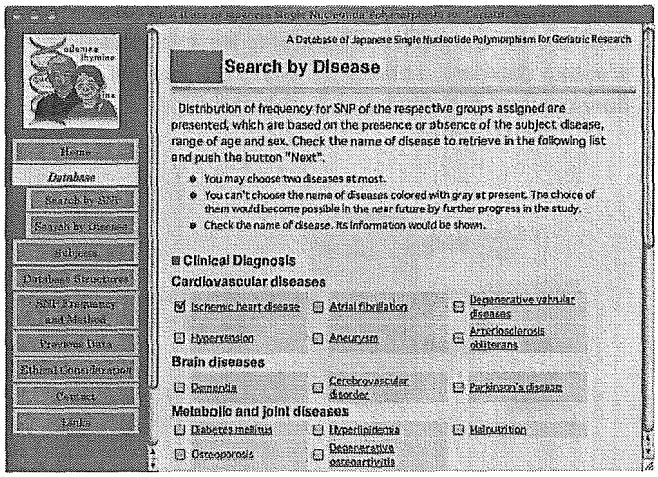

Fig. 2. "Search by Disease" screen of the Japanese SNP database for geriatric research (JG-SNP), which can be seen at http://www.tmgh.metro.tokyo.jp/jg-snp/english/E_top.html.

version: http://www.tmgh.metro.tokyo.jp/jg-snp/japanese/top.html.

4. Discussion

Currently, there are several databases related to genetic polymorphisms that are accessible on the Internet. For example, dbSNP runs under the control of the National Center for Biotechnology Information, USA, and opens at http://www/ncbi.nim.nih.gov/SNP/. A Japanese SNP database (JSNP) has been developed by the Human Genome Center (Institute of Medical Science, University of Tokyo) and the Japan Science and Technology Agency (JST), and opens at http://snp.ims.u-tokyo.ac.jp/index.html. As of December 2003, a huge number of SNPs has been registered: 4,760,000 SNPs under dbSNP and 195,000 SNPs under JSNP. For approximately half of the SNPs in the JSNP, the allele frequency is also available. However, the phenotypes, such as information on individual differences or medical information on the subjects, are not included in these and other SNP databases.

In contrast, the GEAD contains not only the SNP data but also related medical information, and has the following features:

1. Pathological diagnosis is currently recognized as the final diagnosis; thus, the GEAD contains a much more precise diagnosis than the clinical diagnosis.
2. As the entire body is thoroughly examined during autopsy, many undiagnosed diseases such as latent cancers are also discovered and included in the database.
3. The average age of the subject cases, 80 years, matches the average life expectancy of the Japanese people. Therefore, except for some geriatric diseases that occur in only extremely aged people, almost all diseases that may potentially occur during a lifetime are assumed to be included.
4. The GEAD contains many nanogerians and centenarians, which makes it potentially possible to identify the longevity-related genes.

With the progress in the fields of molecular biology and molecular genetics, it has become possible to obtain large amounts of information on the human genome and genetic polymorphisms on the Internet, and management of the information as well as the technology for analysis have been developing at a rapid pace. On the other hand, as for the phenotypic profiles of an individual called the "Phenome" corresponding to the Genome, no consensus has been reached regarding what kind of information should be included in the "Phenome" (Mahner and Kary, 1997; Freimer and Sabatti, 2003; Fredman et al., 2004). Medical information has been frequently chosen in the content of "Phenome", from the perspective of promotion of medical research. In our attempt to create a database for genetic research, the most important data, such as the clinical diagnoses and the pathological findings, were chosen. As the GEAD contains only data obtained from autopsy examinations, the number of subjects is inevitably limited. Thus, it would be necessary to include much more detailed quantitative pathological data for the study of genetic polymorphisms. This issue requires to be solved in future studies.

We have created the JG-SNP database for a wide target audience. The visitors of the JG-SNP website are assumed to be a part of the general public who are interested in geriatric diseases and molecular biology, and researchers on genetic polymorphism. For the general public, the website provides an easy-to-understand explanation on the significance of genetic polymorphisms and brief explanations on each geriatric disease. For researchers on genetic polymorphisms, this database is of the highest value. It is specifically possible for researchers to make comparisons with their own data on SNPs of the candidate genes, and furthermore to obtain the allele frequencies in the elderly population.

In conclusion, we have established an autopsy database for geriatric research, especially focusing on genetic polymorphisms, named GEAD. The GEAD contains both SNP data and medical information derived from autopsy examination of 1074 elderly subjects. Although the number of subjects is limited, it is hoped to open a new field of geriatric research. Based on the GEAD, we have developed another database called the JG-SNP for public use, which is accessible on the Internet.

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